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Bradykinin-activated transmembrane signals are coupled via N_o or N_i to production of inositol 1,4,5-trisphosphate, a second messenger in NG108-15 neuroblastoma–glioma hybrid cells

(GTP-binding proteins/pertussis toxin/adenylate cyclase/ion channels/phosphatidylinositol turnover)

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ABSTRACT **The addition of bradykinin to NG108-15 cells results in a transient hyperpolarization followed by prolonged cell depolarization. Injection of inositol 1,4,5-trisphosphate or Ca^{2+} into the cytoplasm of NG108-15 cells also elicits cell hyperpolarization followed by depolarization. Tetraethylammonium ions inhibit the hyperpolarizing response of cells to bradykinin or inositol 1,4,5-trisphosphate. Thus, the hyperpolarizing phase of the cell response may be due to inositol 1,4,5-trisphosphate-dependent release of stored Ca^{2+} into the cytoplasm, which activates Ca^{2+}-dependent K^+ channels. The depolarizing phase of the cell response to bradykinin is due largely to inhibition of M channels, thereby decreasing the rate of K^+ efflux from cells and, to a lesser extent, to activation of Ca^{2+}-dependent ion channels and Ca^{2+} channels. In contrast, injection of inositol 1,4,5-trisphosphate or Ca^{2+} into the cytosol did not alter M channel activity. Incubation of NG108-15 cells with pertussis toxin inhibits bradykinin-dependent cell hyperpolarization and depolarization. Bradykinin stimulates low K_m GTPase activity and inhibits adenylate cyclase in NG108-15 membrane preparations but not in membranes prepared from cells treated with pertussis toxin. Reconstitution of NG108-15 membranes from cells treated with pertussis toxin with nanomolar concentrations of a mixture of highly purified N_o and N_i [guanine nucleotide-binding proteins that have no known function (N_o) or inhibit adenylate cyclase (N_i)] restores bradykinin-dependent activation of GTPase and inhibition of adenylate cyclase. These results show that [bradykinin·receptor] complexes interact with N_o or N_i and suggest that N_o and/or N_i mediate the transduction of signals from bradykinin receptors to phospholipase C and adenylate cyclase.**

Bradykinin (BK) is a nonapeptide derived by proteolysis of higher molecular weight precursor proteins synthesized in the liver and hypothalamus (1, 2). The pharmacological actions of BK or precursor proteins include muscle contraction, hypertension, pain generation, increase in vascular permeability, and blood coagulation (3–5). However, relatively little is known about the function of BK in the nervous system.

NG108-15 neuroblastoma–glioma hybrid cells have BK receptors (6, 7) and respond to BK by a transient cell hyperpolarization followed by a long-lived depolarization (6, 8) accompanied by an increase in secretion of acetylcholine from NG108-15 cells at synapses with myotubes (8). BK also stimulates phosphatidylinositol turnover in NG108-15 cells (8) and increases cellular levels of inositol 1,4,5-trisphosphate ($InsP_3$) (9) and the release of Ca^{2+} from intracellular stores into the cytoplasm (10).

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In this report we show that pertussis toxin partially inhibits the effects of BK on NG108-15 cells and that activation of BK receptors by BK results in activation of GTPase of N_i (11) or N_o (12) [guanine nucleotide-binding proteins that have no known function (N_o) or inhibit adenylate cyclase (N_i)], which are substrates for pertussis toxin (13, 14). The results suggest that N_i or N_o are involved in signal transduction initiated by [BK·receptor] complexes that result in activation of phospholipase C and inhibition of adenylate cyclase.

METHODS AND MATERIALS

BK was obtained from Sigma or Calbiochem; $InsP_3$ and inositol 2-phosphate were obtained from Sigma; [D-Ala^2,Met^5]enkephalinamide was from Calbiochem. Pertussis toxin was a gift from R. Sekura (National Institute of Child Health). $^{45}CaCl_2$ was obtained from Amersham, and [γ-^{32}P]GTP was from New England Nuclear.

NG108-15 cells used for electrophysiological studies were cultured in polyornithine-coated 35-mm Petri dishes and were treated with 10 μM prostaglandin E_1 and 1 mM theophylline for 1–3 weeks before use (15).

RESULTS

Cell Responses to BK, $InsP_3$, or Ca^{2+}. Addition of BK by iontophoresis to the external surface of an NG108-15 cell resulted in cell membrane hyperpolarization followed by depolarization (Fig. 1*A*) (8). Injection of $InsP_3$ (Fig. 1*B*) or Ca^{2+} (Fig. 1*D*) into the cytoplasm of a cell also resulted in cell hyperpolarization followed by depolarization. Injection of inositol 2-phosphate into the cytoplasm had little or no effect on the cell membrane potential (Fig. 1*C*). These results suggest that the responses of NG108-15 cells to BK are dependent on an increase in cellular levels of $InsP_3$, presumably due to activation of the phospholipase C that catalyzes the formation of $InsP_3$ and diacylglycerol from phosphatidylinositol 4,5-bisphosphate followed by an $InsP_3$-dependent release of stored Ca^{2+} into the cytoplasm.

The relation between the amount of iontophoretic current used for intracellular $InsP_3$ injection and the magnitude of the hyperpolarizing and depolarizing cell responses is shown in Fig. 2*A*. Both responses of cells increased as the quantity of $InsP_3$ iontophoretic currents (A·sec) was increased up to about 65 nC. With some cells, injection of $InsP_3$ with relatively high amounts of iontophoretic current, such as −220 nC, elicited a depolarizing cell response but little or no hyperpolarizing response (Fig. 2 *B* and *C*). The mean hyper-

Abbreviations: BK, bradykinin; $InsP_3$, inositol 1,4,5-trisphosphate; Et_4NCl, tetraethylammonium chloride; N_s or N_i, guanine nucleotide-binding proteins that couple some species of activated receptors with adenylate cyclase stimulation or inhibition, respectively; N_o, an N protein of unknown function.

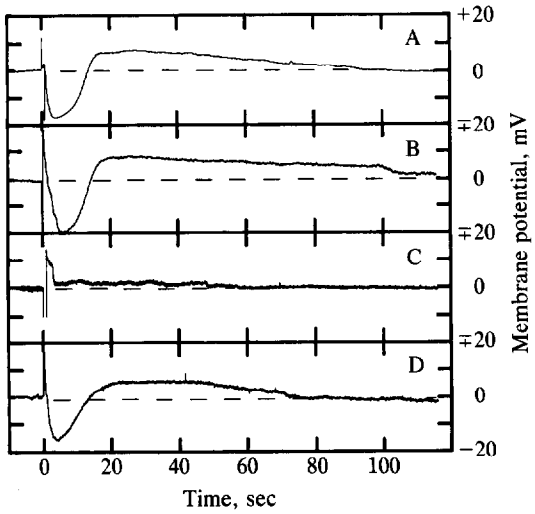

FIG. 1. Typical membrane potential changes of NG108-15 cells evoked by extracellular BK or intracellular injection of InsP_3 or Ca^{2+}. (*A*) Cell hyperpolarization followed by depolarization elicited by extracellular application of BK. The potential changes were recorded with an intracellular microelectrode filled with 3 M KCl. A second micropipette filled with 0.1 mM BK dissolved in 0.1 mM HCl was located extracellularly close to the cell surface. BK was applied at zero time by iontophoresis (50 nA for 1 sec). (*B*) Cell hyperpolarizing and depolarizing responses induced by intracellular injection of InsP_3. The recording electrode (3 M KCl) and a micropipette filled with 1 mM InsP_3 dissolved in H_2O were inserted into an NG108-15 cell. At zero time −100 nA was passed through the InsP_3 pipette for 0.5 sec. (*C*) One mM inositol 2-phosphate dissolved in H_2O was injected intracellularly (−100 nA for 1 sec). (*D*) Ca^{2+} was injected into the cytoplasm of an NG108-15 cell from a micropipette filled with a solution containing 0.5 M $CaCl_2$ by iontophoresis with 100 nA for 1 sec at zero time. The upward and/or downward deflections of the traces between 0 and 2 sec are due to the iontophoretic current rather than the compound applied.

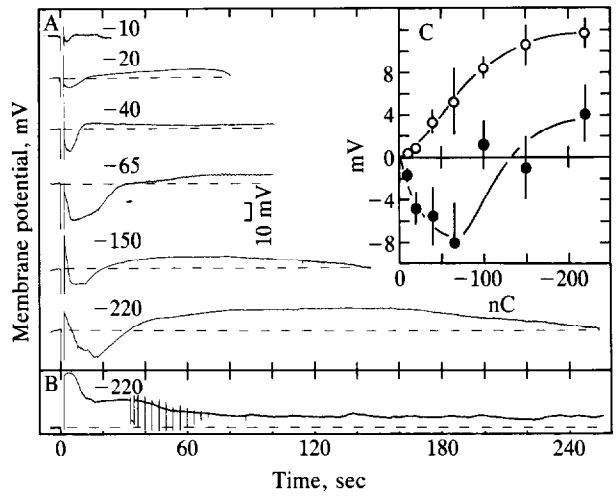

FIG. 2. The relation between the amount of iontophoretic current used for intracellular InsP_3 injection and the magnitudes of the hyperpolarizing and depolarizing cell responses. (*A*) Typical examples of hyperpolarizing and depolarizing responses of NG108-15 cells evoked by intracellular injection of InsP_3 for 2 sec; the amount of current in nC is indicated in each panel. (*B*) An example of InsP_3 intracellular injection by iontophoresis using a large amount of current (−220 nC), which elicited primarily cell depolarization. The discontinuities in the trace at approximately 10–15 sec suggest some cell hyperpolarization. At 30–60 sec passive hyperpolarizing currents were injected to test input membrane resistance. (*C*) The mean changes in membrane potential during the hyperpolarizing and depolarizing phases of NG108-15 cell responses to intracellular injection of InsP_3 with different amounts of iontophoretic current (nC). Each point represents the mean of values obtained from 5–19 cells; each cell was used for only one determination. The hyperpolarization and depolarization values shown are the maximum membrane potential deflections at 5–20 and 30–45 sec, respectively, after injection of InsP_3.

polarizing and depolarizing responses of many cells to injected InsP_3 are shown in Fig. 2*C* as a function of the amount of iontophoretic current applied.

Tetraethylammonium ions (Et_4N^+) completely blocked BK- or InsP_3-dependent cell hyperpolarization but had little or no effect on cell depolarization (Fig. 3*A* and *C*). Et_4N^+ is known to inhibit some Ca^{2+}-dependent K^+ channels (16), which suggests that BK- or InsP_3-dependent hyperpolarization may be due to activation of K^+ channels by Ca^{2+}, thereby increasing the rate of K^+ efflux from the cells. In contrast, Co^{2+} had little effect on the hyperpolarizing response of NG108-15 cells to BK or InsP_3 but inhibited most of the BK- or InsP_3-dependent cell depolarization (Figs. 3 *B* and *D*, respectively). When NG108-15 cells were incubated without Ca^{2+} and with or without 0.1 mM EGTA, the amplitudes of BK-dependent hyperpolarizations and depolarizations were reduced greatly (not shown). Incubation of cells in the absence of Na^+ or in the presence of 1 μM tetrodotoxin had little or no effect on cell responses to BK. The Ca^{2+} channel blocker nifedipine (0.1 μM) had no effect on BK- or InsP_3-dependent hyperpolarizing or depolarizing responses of NG108-15 cells (not shown).

The mean input membrane resistance, determined from the amplitudes of hyperpolarizations induced by repetitive or constant currents applied through the intracellular recording electrode, was reduced to 42 ± 9% (± SEM, n = 5) of the mean control value (12.9 ± 1.1 MΩ, n = 8) during the hyperpolarizing phase of the responses of NG108-15 cells to BK (Fig. 4*A*). The membrane resistance increased to 131 ± 10% (16.8 MΩ) of the control value during the BK-dependent depolarization phase. BK-dependent increases in membrane resistance during the depolarizing phase were found over a wide range of membrane potentials (−80 to −10 mV) (not shown). These results suggest that the addition of BK to NG108-15 cells results in the closure of some ion channels that are open in the absence of BK. In contrast, the average membrane resistance values during the hyperpolarizing and depolarizing phases of the cell response to injection of InsP_3 decreased to 40 ± 6% (n = 6) and 58 ± 7% (n = 18), respectively, of the control value (27.1 ± 2.5 MΩ, n = 14) (Fig. 4 *B* and *C*). The reversal potential for hyperpolarization induced by extracellular application of BK or intracellular injection of Ca^{2+} was −70 to −80 mV, which is close to the K^+ equilibrium potential and suggests that the hyperpolarizing phase is due to the activation of Ca^{2+}-dependent K^+ channels, thereby increasing the rate of K^+ efflux from cells. BK elicited cell hyperpolarization after a delay of 1–2 sec. The delay in cell response was shorter when InsP_3 was injected into the cytosol, and no delay in response was detected when Ca^{2+} was injected. The decreasing delay in cell responses to BK, InsP_3, or Ca^{2+} suggests the following sequential reactions; BK receptor occupancy results in activation of phospholipase C; phosphatidylinositol 4,5-bisphosphate is converted to InsP_3 and diacylglycerol; InsP_3 induces release of stored Ca^{2+} into the cytosol; Ca^{2+}-dependent K^+ channels are opened; and K^+ efflux hyperpolarizes the cell.

Addition of 10–1,000 nM phorbol 12-tetradecanoate 13-acetate or 1-oleoyl-2-acetylglycerol had no effect on the resting membrane potential or on cell responses to BK 0–60 min after addition (not shown). Also, 10 μM A23187 did not mimic the effects of BK on cell membrane potential in control or phorbol ester-treated cells (not shown).

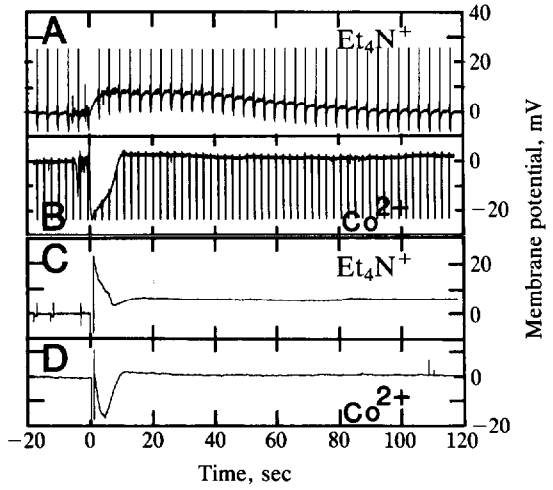

FIG. 3. Effects of Et_4N^+ (*A* and *C*) or Co^{2+} (*B* and *D*) on the responses of NG108-15 cells to extracellular BK (*A* and *B*) or intracellular InsP_3 (*C* and *D*). (*A*) NG108-15 cells were incubated for 20–30 min in a 35-mm Petri dish containing 2 ml of Dulbecco's modified Eagle's medium supplemented with 5 mM Et_4N^+. At zero time 2 μl of a solution containing 10 μM BK dissolved in 150 mM NaCl was added to the surface of the medium close to the cell being tested. Action potentials were evoked by passing depolarizing pulses of current (0.2 nA for 60 msec) through the intracellular recording microelectrode. (*B*) NG108-15 cells were incubated in 2 mM Co^{2+}/20 mM Tris·HCl, pH 7.2/150 mM NaCl/5.4 mM KCl/0.8 mM $MgCl_2$/1.8 mM $CaCl_2$/20 mM glucose for approximately 10–20 min. Hyperpolarizing currents (0.5 nA for 60 msec) were passed through the intracellular recording microelectrode but failed to evoke off-spikes. BK was applied at zero time in a 2-μl drop as described above. (*C*) NG108-15 cells were incubated in the presence of 5 mM Et_4N^+ as described above. InsP_3 (0.1 mM) was injected into the cytoplasm by iontophoresis (−100 nA for 1 sec). (*D*) Cells were incubated in the presence of 2 mM Co^{2+} in the medium as described above for 10–20 min. InsP_3 was injected intracellularly at zero time (−200 nA for 1 sec).

BK-Dependent Efflux of $^{45}Ca^{2+}$. As reported (8), BK stimulates $^{45}Ca^{2+}$ influx into NG108-15 cells. The effect of BK on $^{45}Ca^{2+}$ efflux from NG108-15 cells is shown in Fig. 5. Cells were incubated in the presence of 1.8 mM $^{45}CaCl_2$ for 8 min to promote $^{45}Ca^{2+}$ uptake and washed for 3 min, and then dishes were perfused with control medium or medium with BK. BK stimulated the rate of $^{45}Ca^{2+}$ efflux approximately 3-fold during the first 20 sec, but by 80 sec the rate of $^{45}Ca^{2+}$ efflux had returned to the control value.

Effects of Pertussis Toxin on Cell Responses to BK. Both the hyperpolarizing and depolarizing responses of NG108-15 cells to BK were inhibited by treatment of the cells with pertussis toxin (200 ng per ml, 15 hr) (Fig. 6). The maximum inhibition by pertussis toxin was obtained with 1 μM BK; higher concentrations of BK resulted in less inhibition by pertussis toxin. The maximum inhibition of hyperpolarization (72%) was obtained with 1000 ng of pertussis toxin per ml, the highest concentration tested; 50% of the maximum observed inhibition was obtained with 35–100 ng of pertussis toxin per ml of medium. These results suggest that GTP-binding regulatory proteins such as N_i (13, 14) or N_o (12) mediate the hyperpolarizing cell response to BK. The results also are consistent with the suggestion that pertussis toxin acts primarily to decrease receptor affinity for ligands (17).

Because all known GTP-binding regulatory proteins also display hormone-stimulated GTPase activities, we measured the effect of BK on GTPase activity of NG108-15 cell membranes. The data shown in Table 1 demonstrate that BK or the opioid peptide [D-Ala^2,Met^5]enkephalinamide stimulated the activity of a low K_m GTPase in NG108-15 membranes and that the stimulatory effects of BK or [D-Ala^2,Met^5]enkephalinamide were inhibited by treating the cells with pertussis toxin before the membranes were prepared. BK-dependent stimulation of low K_m GTPase activity was restored to membranes of pertussis toxin-treated

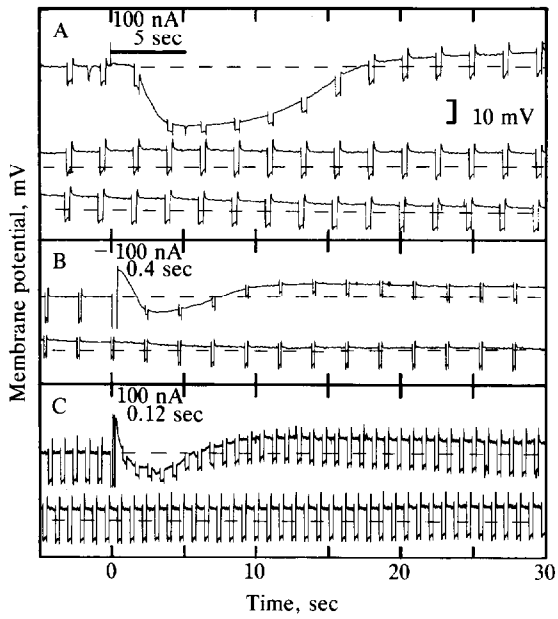

FIG. 4. Changes in membrane resistance elicited by BK iontophoresis (*A*), InsP_3 injection (*B*), or Ca^{2+} injection (*C*). (*A*) Membrane resistance was monitored by passing repetitive hyperpolarizing pulses of current (0.5 nA for 400 msec) through the intracellular recording microelectrode. BK was applied extracellularly by iontophoresis (100 nA for 5 sec). The amplitudes of the hyperpolarizing pulses are measures of membrane resistance. (*B*) InsP_3 was injected intracellularly by iontophoresis (−100 nA for 400 msec). Membrane resistance was measured by passing hyperpolarizing pulses (0.2 nA for 200 msec) through the intracellular recording microelectrode. (*C*) Ca^{2+} was injected intracellularly by iontophoresis (100 nA for 120 msec). Repetitive hyperpolarizing pulses of current (0.2 nA for 300 msec) were passed through the recording intracellular microelectrode.

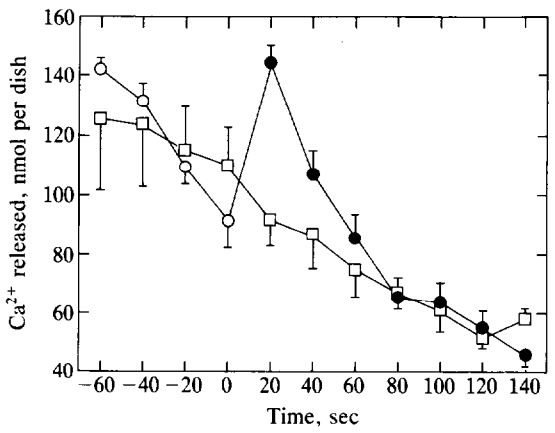

FIG. 5. BK-stimulated $^{45}Ca^{2+}$ efflux from NG108-15 cells. The cells were incubated in the medium described in the legend to Fig. 3*B* without Co^{2+} for 2 min and then for 8 min at room temperature in medium supplemented with 4 μCi (1 Ci = 37 GBq) of $^{45}CaCl_2$, washed three times with medium without $^{45}Ca^{2+}$, and then perfused for 2.9 min. At zero time cells were perfused with medium that contained 1 μM BK (●) or medium without BK (□). Fractions (1 ml) were collected at 20-sec intervals. Each point represents the mean of 3–4 values ± SEM. Each Petri dish contained approximately 5 × 10^5 cells.

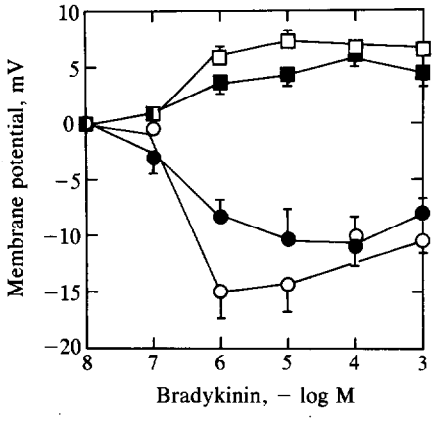

FIG. 6. The effects of pertussis toxin on the hyperpolarizing (●, ○) and depolarizing (■, □) responses of NG108-15 cells grown in dishes that were not coated with polyornithine. Droplets (2 μl each) of a solution containing the indicated concentration of BK dissolved in 150 mM NaCl were applied to the surface of the medium near the cell that was being tested. Each point represents the mean value of responses of nine cells. Cells were incubated for 15 hr with 0.34 mM ammonium sulfate (control cells) or with 200 ng of pertussis toxin/ml of medium and 0.34 mM ammonium sulfate before being tested. ○, Hyperpolarizing responses of control cells to BK; ●, BK-dependent hyperpolarizing responses of NG108-15 cells that had been treated with pertussis toxin; □, depolarizing responses of control cells to BK; ■, BK-dependent depolarizing responses of cells that had been treated with pertussis toxin.

NG108-15 cells by the addition of a mixture of bovine brain N_o and N_i (approximately 70% N_o and 30% N_i) estimated to be >95% pure (19) (Fig. 7*A*); half-maximal stimulation of GTPase by BK was obtained with <1 nM N_o/N_i. As reported earlier (19) and as shown in Fig. 7*A*, the N_o/N_i mixture also reconstituted a [D-Ala^2,Met^5]enkephalinamide-dependent, opiate receptor-mediated stimulation of GTPase. Interestingly, BK-stimulated GTPase activity was reconstituted at appreciably lower concentrations of the N_o/N_i mixture than those needed for opiate-stimulated GTPase. We found also that BK stimulation of GTPase activity was diminished only slightly in the presence of a saturating concentration (10 μM) of [D-Ala^2,Met^5]enkephalinamide (Fig. 7*B*). Such additivity of effects suggests that BK receptors may interact preferentially with N_o, and opiate receptors may interact preferentially with N_i. Additivity of the effects of BK and opiates is confirmed by the observation that BK-dependent inhibition of adenylate cyclase still was observed in the presence of saturating concentrations of opiates (Fig. 7*C*). Ligands for other species of NG108-15 receptors that mediate inhibition of adenylate cyclase, such as norepinephrine, which activates α_2-adrenergic receptors (21), or somatostatin (22), no longer inhibit adenylate cyclase or stimulate low K_m GTPase in the presence of saturating concentrations of opiates. Thus, the mechanism of coupling BK receptors to inhibition of adenylate cyclase differs from the mechanism coupling α_2-receptors or somatostatin receptors to inhibition of adenylate cyclase.

Table 1. Attenuation of peptide-stimulated GTPase by pertussis toxin

Addition	P_i formed, pmol/min per mg of protein	
	Control cells	Pertussis toxin-treated cells
Water	26.0 ± 1.5	19.9 ± 1.6
BK (10 μM)	32.6 ± 1.8	18.9 ± 1.8
[D-Ala^2,Met^5]-enkephalinamide (10 μM)	38.0 ± 2.3	15.7 ± 1.6

Attenuation of peptide-stimulated low K_m GTPase activity by pertussis toxin. Membranes prepared from NG108-15 cells were incubated with or without 20 ng of pertussis toxin per ml of medium for 22 hr. Low K_m GTPase activity was assayed as described (18).

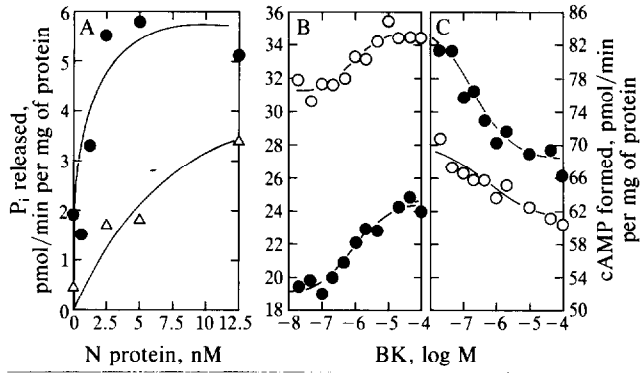

FIG. 7. (*A*) Reconstitution of BK-stimulated (●) or opiate-stimulated (△) low K_m GTPase activities of membranes prepared from pertussis toxin-treated NG108-15 cells. Membranes were incubated with the indicated concentrations of a mixture of bovine brain N_i and N_o estimated to be >95% pure for 15 min at 30°C in the standard assay mixture (18) without radioactive GTP and then were incubated for an additional 15 min in the presence of approximately 60,000 cpm (1 μM) [γ-^{32}P]GTP. $^{32}P_i$ released in the absence of BK or [D-Ala^2,Met^5]enkephalinamide has been subtracted. Mean values (three experiments) are shown. (*B*) Low K_m GTPase activity of membranes prepared from untreated NG108-15 cells is shown as a function of BK concentration. ○, BK with 10 μM [D-Ala^2,Met^5]enkephalinamide; ●, BK without [D-Ala^2,Met^5]enkephalinamide. The ordinate scale is pmols of P_i released per min/mg of protein. (*C*) Adenylate cyclase activity of membranes prepared from untreated NG108-15 cells, assayed as described (20), is shown as a function of BK concentration in the absence (●) or presence (○) of 10 μM [D-Ala^2,Met^5]enkephalinamide.

DISCUSSION

Exposure of NG108-15 cells to BK elicits increases in InsP_3 and diacylglycerol levels (8); in turn, InsP_3 stimulates the release of stored Ca^{2+} into the cytoplasm (10), thereby activating Ca^{2+}-dependent K^+ channels and increasing the rate of K^+ efflux. Cell hyperpolarization results from the increase in K^+ efflux.

The observations that support these conclusions are: (*i*) injection of InsP_3 or Ca^{2+} into the cytoplasm of NG108-15 cells results in cell hyperpolarization followed by depolarization; (*ii*) hyperpolarization of cells elicited by BK, InsP_3, or Ca^{2+} is accompanied by a decrease in membrane resistance—i.e., an increase in cell permeability to ions; (*iii*), the reversal potential for BK-dependent hyperpolarization is approximately −80 mV, which is close to the equilibrium potential for K^+; (*iv*) the amplitudes of the hyperpolarizing responses of 108CC-25 (23) and NG108-15 cells (unpublished results) to BK are dependent on the concentration of extracellular K^+; and (*v*) BK-dependent hyperpolarization is blocked by Et_4N^+, which is known to inhibit some Ca^{2+}-dependent K^+ channels (16).

The long-lasting depolarization evoked by BK is associated with an increase in membrane resistance—i.e., cell permeability to ions decreases. Other species of receptors have been shown to mediate cell depolarization by decreasing cell permeability, such as muscarinic acetylcholine receptors (24) and receptors for luteinizing hormone-releasing hormone (25—27), substance P (28, 29), thyrotropin-releasing hormone (30, 31), neurotensin (32), somatostatin (33), angiotensin (34), prostaglandin D_2 (35), and the small cardioactive

peptide (36). Cell depolarization mediated by muscarinic acetylcholine receptors has been attributed to inhibition of M channels for K^+, which decreases K^+ efflux from cells (24). Recent results obtained with voltage-clamp conditions show that NG108-15 cells have M channels that are inhibited by BK (Higashida and Brown, unpublished data).

In contrast, injection of InsP_3 or Ca^{2+} into the cytoplasm of NG108-15 cells results in cell hyperpolarization followed by a long-lasting depolarization that is accompanied by a decrease in membrane resistance—i.e., an increase in cell permeability to ions. Injection of InsP_3 or Ca^{2+} into the cytoplasm did not affect the activity of M channels; therefore, elevation of cytosolic levels of InsP_3 or Ca^{2+} elicited most, but not all, of the cell responses to extracellular BK. Because BK stimulates the formation of InsP_3 in NG108-15 cells, part of the BK-induced depolarization probably also is due to an increase in cell permeability to ions. The most likely candidates for the ion channels that are activated during the depolarizing phase are the Ca^{2+}-dependent cation channel (37) and a Ca^{2+} channel.

We find that BK-dependent cell hyperpolarization and depolarization are inhibited by pertussis toxin, which suggests that a GTP-binding regulatory protein such as N_o or N_i plays a role in the signal transduction process. BK was shown to stimulate a low K_m GTPase and to inhibit adenylate cyclase in NG108-15 membrane preparations, and these effects of BK were blocked by pertussis toxin. Reconstitution of membranes from pertussis toxin-treated NG108-15 cells with nanomolar concentrations of a mixture of highly purified bovine brain N_o and N_i restored BK-dependent activation of low K_m GTPase and inhibition of adenylate cyclase. BK- and [D-Ala^2,Met^5]enkephalinamide-dependent inhibitions of adenylate cyclase were additive, whereas norepinephrine- and [D-Ala^2,Met^5]enkephalinamide-dependent inhibitions were not additive, which suggest that BK and opiates inhibit adenylate cyclase by different mechanisms. Because the $[\beta\cdot\gamma]$ subunits of the N proteins are functionally interchangeable (38), activation of any N protein, with dissociation of $[\alpha\cdot\beta\cdot\gamma]$ complexes to α and $[\beta\cdot\gamma]$ will result in inhibition of adenylate cyclase by mass action, since the released $[\beta\cdot\gamma]$ subunits will combine with the free α subunit of activated N_s, the N protein that stimulates adenylate cyclase. In addition, our results suggest that N_o or N_i are involved in the transduction of signals from BK receptors to Ca^{2+} mobilization, presumably via activation of phospholipase C, as has been suggested for several other species of receptors (39–42).

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